Genetics and Molecular Biology, 26, 4, 537-543 (2003)

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Research Article

Comparative molecular analysis of *Herbaspirillum* strains by RAPD, RFLP, and 16S rDNA sequencing

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Abstract

Herbaspirillum spp. are endophytic diazotrophic bacteria associated with important agricultural crops. In this work, we analyzed six strains of *H. seropedicae* (Z78, M2, ZA69, ZA95, Z152, and Z67) and one strain of *H. rubrisubalbicans* (M4) by restriction fragment length polymorphism (RFLP) using *Hin*dIII or *Dra*I restriction endonucleases, random amplified polymorphic DNA (RAPD), and partial sequencing of 16S rDNA. The results of these analyses ascribed the strains studied to three distinct groups: group I, consisting of M2 and M4; group II, of ZA69; and group III, of ZA95, Z78, Z67, and Z152. RAPD fingerprinting showed a higher variability than the other methods, and each strain had a unique electrophoretic pattern with five of the six primers used. Interestingly, *H. seropedicae* M2 was found by all analyses to be genetically very close to *H. rubrisubalbicans* M4. Our results show that RAPD can distinguish between all *Herbaspirillum* strains tested.

Key words: Herbaspirillum, RAPD, RFLP, Phylogeny, 16S rDNA.

Received: December 9, 2002; Accepted: August 10, 2003.

Introduction

Herbaspirillum spp. are a group of β-Proteobacteria (Baldani *et al*., 1986; Gillis *et al*., 1990) comprising three species: *H. seropedicae* (Baldani *et al*., 1986), *H. rubrisubalbicans* (Baldani *et al*., 1996) and *H. frisingense* (Kirchhof *et al*., 2001). These bacteria are endophytic diazotrophs capable of colonizing plant tissues of gramineae such as rice, maize, sugar cane, sorghum, banana, and pineapple (Olivares *et al*., 1993; Baldani *et al*., 1986, 1992; Cruz *et al*., 2001). Inoculation with *Herbaspirillum* spp. can improve plant growth and productivity (Baldani *et al*., 1995). These effects are attributed in part to the nitrogen fixed by the bacteria (Döbereiner and Pedrosa, 1987; Döbereiner 1991; Döbereiner *et al*., 1995; Boddey *et al*., 1995; Olivares *et al*., 1996) and also to the production of phytohormones, such as auxins and gibberillins, which can induce plant growth (Bastián *et al.*, 1998). However, evaluation of the bacterial contribution to the associated plant under field conditions is complicated by the presence, in both soil and seeds, of other bacteria which can associate with the crop (Barraquio *et al*., 1997). The recovery and identification of inoculated strains is essential to correlate gains in productivity with successful colonization.

In recent years, much progress has been made in the development of molecular tools to identify bacteria, such as restriction fragment length polymorphism (RFLP), 16S ribosomal DNA (rDNA) sequencing, and random amplified polymorphic DNA (RAPD).

In this work, we analyzed six strains of *Herbaspirillum seropedicae* (Z78, M2, ZA69, ZA95, Z152 and Z67) and one strain of *Herbaspirillum rubrisubalbicans* (M4) by a combination of methods for genetic differentiation, including RFLP, RAPD, and comparison of partial sequences of the 16S rRNA genes.

Materials and Methods

Strains and culture conditions

The *Herbaspirillum seropedicae* strains M2, ZA69, ZA95, Z152, Z78 and Z67, and the *H. rubrisubalbicans* strain M4 used in this study were grown overnight in 50 mL NFbHP-malate (Klassen *et al*., 1997) at 30 °C in a rotary shaker.

RAPD fingerprinting

Genomic DNA of *Herbaspirillum* strains was purified as follows: 5 mL of the overnight cultures were centrifuged, and the pellet was suspended in 500 µL TES buffer

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(50 mmol/L Tris-HCL, 20 mmol/L EDTA, and 100 mmol/L NaCl, pH 8.0), treated with 100 µg of lysozyme, and incubated at 30 °C for 3 h. Cells lysis was completed by adding 50 µL SDS (10%), followed by incubation at room temperature for 5 min and treatment with proteinase K (100 µg/mL) at 37 °C overnight. Purified DNA was obtained by three successive extractions with equilibrated phenol (1 vol) and one with chloroform: isoamyl alcohol (24:1). The genomic DNA was precipitated with 2 volumes of absolute ethanol, washed with 70% ethanol, vacuum-dried, and suspended in 200 µL of $T_{10}E_1$ (10 mM Tris, 1 mM EDTA, pH 8.0).

Ten ng of genomic *Herbaspirillum* DNA were submitted to random amplification using the Ready·To·Go™ RAPD Analysis Beads Kit (Amersham Biosciences) according to the manufacturer's instructions. Six different primers, previously described for characterization of bacterial strains (Amersham Biosciences), were used in the RAPD reactions (Table 1). The electrophoretic profiles were scored according to the presence (1) or absence (0) of a particular band, generating a binary matrix. Similarity of all pair-wise combinations of the numerical profiles was determined by Dice's coefficient (Dice, 1945) and clustered by unweighted pair-group analysis using arithmetical averages (UPGMA) (Sokal and Michener, 1958). A dendrogram was constructed from the patterns, using the NTSYS pc 2.0 program (Exeter Software - scientific software for teaching and research - http://www.exetersoftware.com/index.html). All samples and a negative control without DNA were run in parallel in the same thermocycler. At least three independently amplified fragment patterns of each sample were obtained to confirm the result.

RFLP fingerprinting

Genomic DNA isolated as described above was digested with *Hin*dIII or *Dra*I (Invitrogen) and submitted to agarose gel (0.7%) electrophoresis. Analyses were performed as described for RAPD fingerprinting.

DNA sequencing of 16S rDNA

The 16S rDNA of the *Herbaspirillum* strains was amplified using primers Y1 and Y3 as previously described (Cruz *et al*., 2001). Briefly, diluted overnight cultures (1:10) were boiled for 10 min and cooled on ice. Reaction mixtures (25 µL) contained 10 µL of boiled cultures, *Taq* DNA polymerase buffer (50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl, pH 9.0), 200 µmol/L of each deoxynucleotide, 0.4 µmol/L of each primer, and 1U of *Taq* DNA polymerase (Invitrogen). The mixtures were incubated at 93 °C for 2 min, followed by 34 amplification cycles of 93 °C for 45 s, 62 °C for 30 s, and 72 °C for 2 min each. Conserved regions of 16S rDNA from subclasses alpha, beta and gamma of the Proteobacteria were identified from the alignment of 65 sequences from the GenBank database, and used to design the primers 16S362f, 16S786f, 16S1203f, 16S1110r, and 16S805r (Table 1). DNA sequences of the amplified products were determined using dye terminator chemistry in an ABI Prism 377 automated DNA sequencer (Applied Biosystems). The 16S rDNA sequences obtained were deposited in the GenBank database, and their accession numbers are in parentheses: *H. seropedicae* strains M2 (AY191276), ZA69 (AY191272), ZA95 (AY191274), Z152 (AY191273), Z78 (AY191275).

DNA Sequencing analysis

DNA sequences were assembled and edited using the BioEdit package (Hall, 1999) and aligned by the Contig As-

Table 1 - Sequences of the primers used.

Primers	Sequence (5'→3')	Reference
Y1	TGGCTCAGAACGAACGCTGGCGGC	Young *et al*., 1991
Y2	ACTCCTACGGGAGGCAGCAGTGGG	Young *et al*., 1991
Y3	CTGACCCCACTTCAGCTTGTTCCAT	Cruz *et al.*, 2001
16S362f	CTCCTACGGGAGGCAGTGGGG	this work
16S786f	CGAAAGCGTGGGGAGCAAACAGG	this work
16S1203f	GAGGTGGGGATGACGTCAAGTCCTC	this work
16S1110r	TGCGCTCGTTGCGGGACTTAACC	this work
16S805r	GACTACCAGGGTATCTAATCCTG	this work
RAPD Primer 1	GGTGCGGGAA	Amersham Biosciences
RAPD Primer 2	GTTTCGCTCC	Amersham Biosciences
RAPD Primer 3	GTAGACCCGT	Amersham Biosciences
RAPD Primer 4	AAGAGCCCGT	Akopyanz *et al*., 1992
RAPD Primer 5	AACGCGCAAC	Akopyanz *et al*., 1993
RAPD Primer 6	CCCGTCAGCA	Akopyanz *et al*., 1994

sembly Program (CAP) (Huang, 1992). The phylogenetic tree was obtained by a neighbor-joining method, using the MEGA 2 software (Kumar *et al*., 2001).

Results and Discussion

The total DNA restriction patterns of the *Herbaspirillum* strains were used to construct a dendrogram (Figure 1A), which clustered them into three groups (Figure 1A and Table 2). The *Hin*dIII or *Dra*I restriction profiles of M2 and M4 were identical, as were those of *H. seropedicae* Z67 and Z78, thus defining two electrophoretic types. Strains ZA95 and Z152 had identical patterns with *Dra*I, but *Hin*dIII produced two different bands. However, analyses of their electrophoretic profiles positioned these strains together with Z67 and Z78, forming a cluster with a similarity coefficient of 100%. *H. seropedicae* ZA69 had a distinct electrophoretic pattern and was allocated into a separate group, closer to the M2/M4 cluster. These results indicate that, as far as the separation of the *Herbaspirillum* strains is concerned, restriction length polymorphism has a limited potential.

Genomic diversity of the *Herbaspirillum* strains was also investigated by random amplified polymorphic DNA (RAPD) analysis. Each of the six primers used generated electrophoretic DNA patterns for the strains studied (Table 2 and Figure 2). The analysis of these patterns produced highly congruent DNA fingerprint clustering, in overall agreement with the RFLP results (Figure 1A). With the exception of primer 1, all primers produced unique patterns for M2 and M4, allowing unequivocal differentiation of these strains. As with RFLP, analysis of the RAPD fingerprinting patterns revealed three main clusters of strains (Figure 1B), with a similarity level of approximately 39%. Cluster I was formed by M2 and M4, with a similarity level of 65%. Strain ZA69 was located in a separate branch, closer to M2 and M4, but with a similarity level of 40%. Strains ZA95, Z78, Z152, and Z67 were located in a separate cluster, occupying distinct positions in the dendrogram and forming two sub-groups, comprising strains ZA95 and Z78 (72% similarity), and Z152 and Z67 (65% similarity), respectively. Cluster analysis of RAPD profiles supported the differences noted by visual observation of the electrophoretic profiles (data not shown). Furthermore, the profiles obtained by RAPD showed a higher level of variability than those obtained by RFLP, since, using primers 2 to 6, all strains could be distinguished by at least one band (Table 2). These results show that RAPD is the most sensitive and convenient method tested to unequivocally identify *Herbaspirillum* strains using whole genomic DNA. However, since low reproducibility has been attributed to RAPD profiling, this may reduce its potential application (Penner *et al*., 1993; Wang *et al*., 1993). To minimize this effect, we used the same procedure for DNA purification from all strains and a commercially available RAPD system with standardized components and conditions, which was reliable and reproducible in this study. The Ready-To-Go (Amersham Biosciences) beads format also significantly reduced the number of pipetting steps, thereby increasing the reproducibility of the RAPD technique.

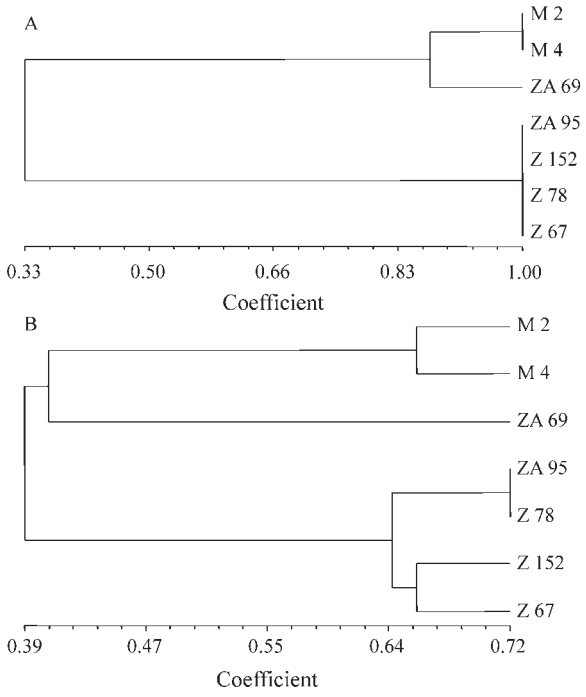

Figure 1 - **Panel A** – Dendrogram inferred from the RFLP profiles obtained by digestion of total DNA of *Herbaspirillum* strains with restriction endonucleases *Hin*dIII or *Dra*I. **Panel B** – Dendrogram inferred from RAPD profiles of the *Herbaspirillum* strains. Similarities were calculated using Dice's coefficient, and clustering was achieved by UPGMA (Sokal and Michener, 1995).

Table 2 - Genotypic characterization of *Herbaspirillum* spp., as revealed by RAPD and RFLP profiling and 16S rDNA sequence analysis.

Organism	RAPD pattern[a]	RFLP pattern[b]	16S rDNA[c]
H. seropedicae			
M2	aaaaaa	aa	a
ZA69	cccccc	bb	b
ZA95	dddddd	cc	c
Z152	deeeee	cd	c
Z67	efffff	ce	c
Z78	fggggg	ce	c
H. rubrisubalbicans			
M4	bbbbbb	aa	a

[a]RAPD patterns obtained with different primers. Each letter defines a common pattern for primers 1 to 6, respectively (see Table 1).
[b]RFLP patterns obtained with restriction endonucleases *Hin*dIII and *Dra*I, respectively. Each letter defines a common pattern.
[c]The Y1-Y3 region of the 16S rDNA covers approximately 1500 bp. The letters represent genotypes in the same cluster.

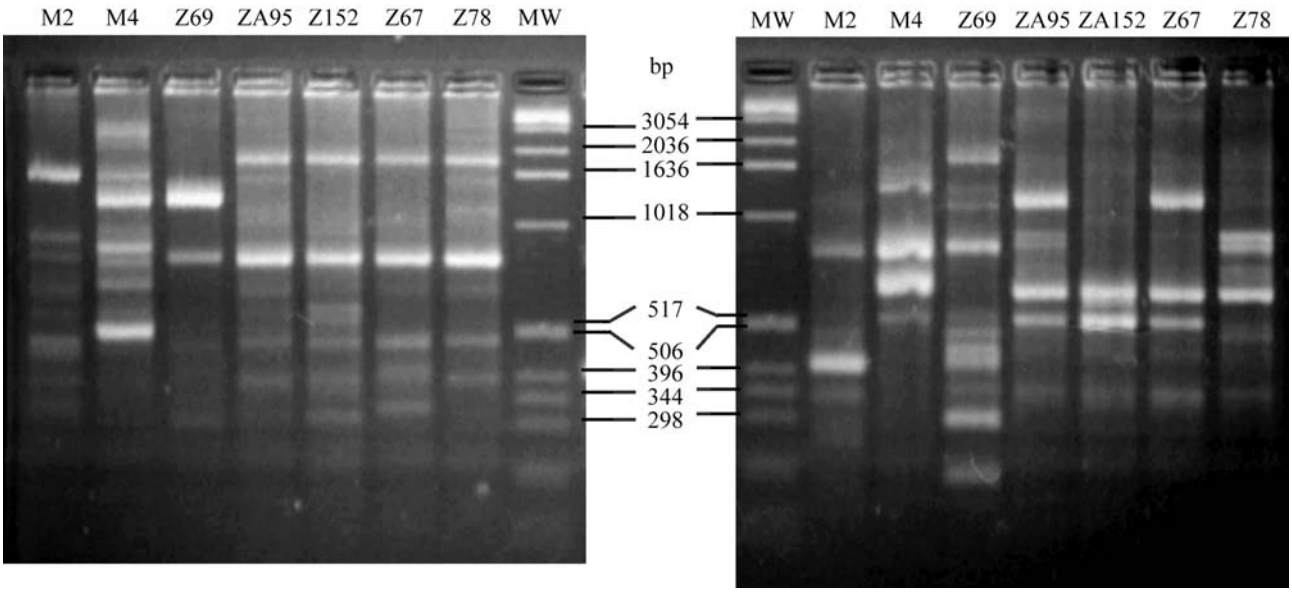

Figure 2 - Agarose gel (2.0%) electrophoresis of RAPD profiles of the *Herbaspirillum seropedicae* (M2, Z69, ZA95, ZA152, Z67 and Z78) and *Herbaspirillum rubrisubalbicans* (M4) strains obtained with primers 3 (left) or 4 (right). MW indicates the molecular weight markers in bp (1 kb Ladder, Invitrogen). DNA was stained with ethidium bromide (0.5 μg/mL).

Lasker (2002) recently reported that repeated runs of the same or different DNA preparations of the same strain of *Aspergillus fumigatus* produced highly reproducible results using RFLP, RAPD, sequence-specific DNA primer (SSDP) analysis or polymorphic microsatellite markers (PMM) analysis (Lasker, 2002). Coenye *et al*. (2002) reached similar conclusions by analyzing *Burkholderia cepacia* Genomovar III isolates, using pulsed-field gel electrophoresis (PFGE), BOX-PCR fingerprinting and random amplified polymorphic DNA (RAPD) typing.

Zhang *et al*. (2002) comparing typing methods to differentiate *Streptococci* Group B showed that RAPD was a useful assay for the rapid characterization of these strains and was more discriminatory than conventional serological assays. These authors also reported that the RAPD assay was faster, more convenient and easier to perform than alternative DNA analytical procedures such as pulsed-field gel electrophoresis. In addition, van den Braak *et al*. (2002) obtained highly congruent DNA fingerprint clustering of vancomycin-resistant enterococci (VRE), employing well-standardized RAPD or PFGE protocols.

The 16S rDNA from all *Herbaspirillum* strains used in this work was amplified using primers Y1 (Young *et al*., 1991) and Y3 (Cruz *et al*., 2001), which allowed the amplification of the nearly complete 16S rRNA genes. The PCR products were thoroughly sequenced in both orientations, yielding the complete sequence of the Y1-Y3 fragment with a three-times redundancy. The partial sequence of the 16S rRNA gene of the *H. seropedicae* strain Z67, deposited in the GenBank database (Kirchhof *et al*., 2001), differed from the sequence obtained in this study by three bases. The 16S rDNA sequence of the *H. rubrisubalbicans* strain M4 deposited in GenBank (Kirchhof *et al*., 2001) was identical to that determined in this work.

Ribosomal operons are of great relevance for the study of bacterial evolution and phylogeny (Woese, 1987), and sequencing of 16S rDNA has been widely used to re-construct phylogenetic relationships of microorganisms (Gutell *et al*., 1994; Luz *et al*., 1998).

Phylogenetic analysis of the partial 16S rDNA sequences from the *Herbaspirillum* strains studied here, together with related sequences deposited in GenBank (Figure 3), positioned the *Herbaspirillum* species (*H. seropedicae*, *H. rubrisubalbicans* and *H. frisingensis*), *Herbapirillum*-like isolates, and the related species *Pseudomonas huttiensis* in separate branches, well supported by the bootstrap values (see Figure 3). Within the *H. seropedicae* branch, strains Z67, Z78, ZA95, and Z152 formed a tight cluster with a bootstrap value of 96. Strain ZA69 formed a separate cluster in the same branch, consistent with its taxonomic position. Strains *H. seropedicae* M2 and *H. rubrisubalbicans* M4 had very similar 16S rDNA sequences, and grouped in the *H. rubrisubalbicans* branch with a bootstrap value of 89. Although strains M2 and M4 belong to different species, as determined by physiological assays (Baldani *et al*., 1996), the analyses carried out in this work showed very close genetic proximity.

The genetic proximity between strains of the species *H. seropedicae* and *H rubrisubalbicans* was also observed by Cruz *et al*. (2001), using ARDRA (amplified rDNA restriction analysis) and sequencing of approximately 300pb of the 16S rDNA, thereby highlighting the low level of variability of these organisms. Kirchhof *et al.* (2001) and Caballero-Mellado and Martinez-Romero (1994) evaluating

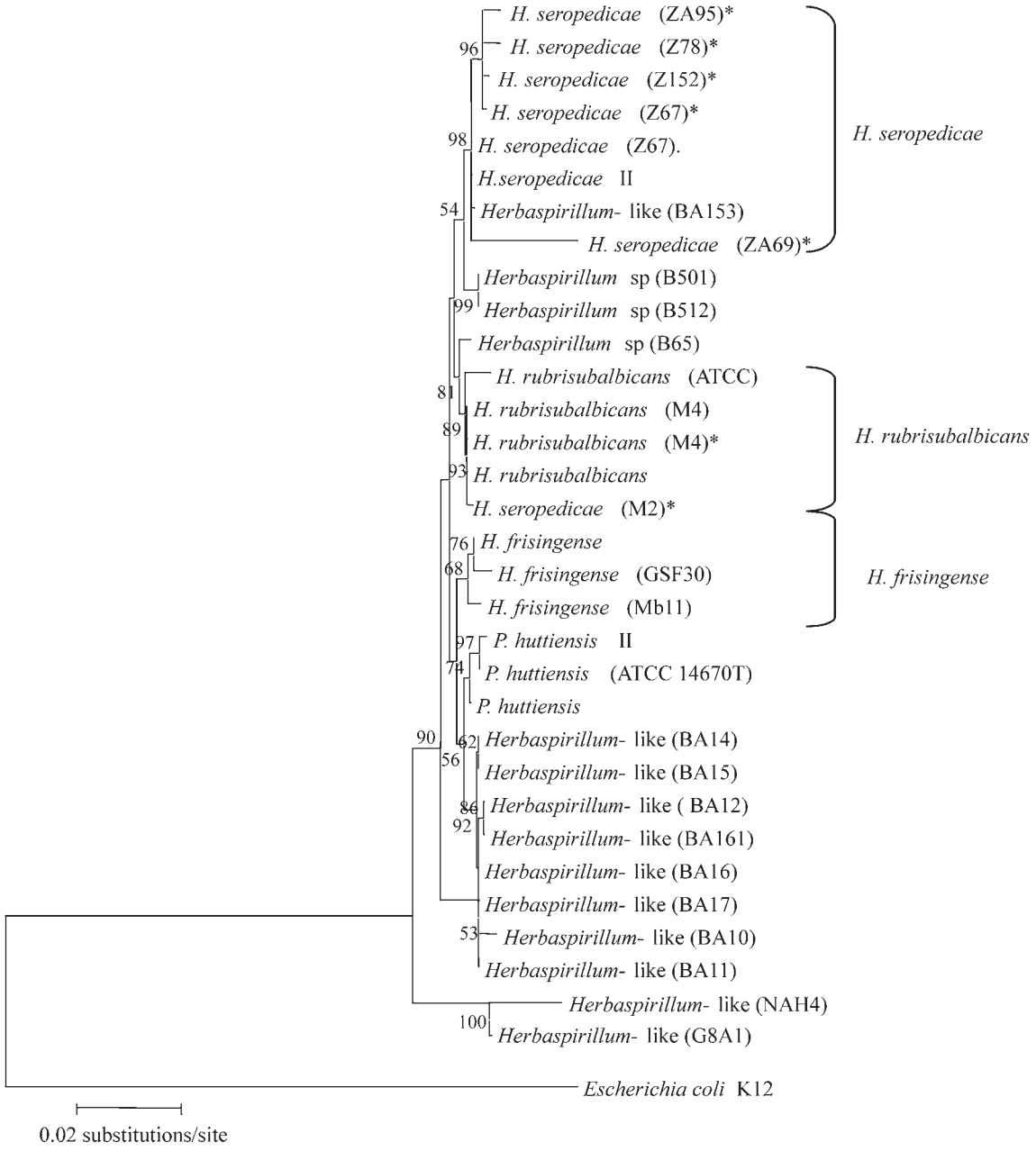

Figure 3 - Phylogenetic tree inferred from 16S rDNA sequences of *Herbaspirillum* strains obtained in this work (*) or available in the GenBank database, and representative beta-Proteobacteria. The tree was constructed by the neighbor-joining method. The boot-strap values were calculated from 2000 trees, and each number on a branch indicates the percentage of trees in which the node was supported. The bar represents the percentage of sequence divergence.

Herbaspirillum frisingense and *Gluconacetobacter diazotrophicus* strains, also concluded that *Herbaspirillum* strains formed a group of tight genetic relationships and low variability.

Herbaspirillum spp. are endophytic diazotrophs with low survival in plant-free soils (Baldani *et al*., 1992), suggesting a strong adaptation of these bacteria to the endophytic state. McArthur *et al.* (1988) suggested that close genetic proximity may be accounted for by adaptation to very stable habitats for long periods.

In summary, molecular tools evaluating either the 16S rRNA gene or the entire genome (RAPD and RFLP) allowed distinction of the seven *Herbaspirillum* strains studied. RAPD profiling using a commercially available system unequivocally differentiated all the strains tested and proved very reliable. This finding agrees with the observation that the 16S rDNA sequences are highly conserved among the species and potentially useful to distinguish between distantly related organisms (Woese, 1987). In contrast, RAPD utilizes fragment amplification of the whole

genome, therefore being well suited to detect differences between closely related organisms.

Acknowledgments

We thank Roseli Prado, Valter Baura and Julieta Pie for technical assistance. CNPq/PRONEX/Fundação Araucária/Paraná Tecnologia supported this work.

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Editor: Darcy Fontoura de Almeida